PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

in re: Anagnostou, et al.

Serial No.: 09/525,808 Filed: March 15, 2000 Group Art Unit: 1642

Examiner: Christopher H. Yaen

For: METHOD OF TREATING ENDOTHELIAL INJURY

April 15, 2003

Commissioner for Patents Washington, DC 20231

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DECLARATION UNDER 37 C.F.R § 1.132 OF GEORGE SIGOUNAS, Ph.D.

TECH CENTER 1600/2900

Sir:

I, George Sigounas, Ph.D., do hereby declare and say as follows:

- 1. I received my Ph.D. from Boston University in Cellular Biology. I am currently Professor of Medicine at East Carolina University School of Medicine in Greenville, North Carolina. I am a co-inventor on the above-identified patent application.
- 2. The following experiments were carried out under my direction. These experiments were designed to assess the ability of erythropoietin (EPO) to effectively prevent or protect and/or repair injuries of the endothelium caused by chemotherapeutic drugs *in vivo*.
- 3. To perform the *in vivo* studies, we used an established mouse model of bleomycin-induced toxicities to investigate the effects of EPO on structural and functional changes in endothelium and endothelial cells caused by bleomycin.

Female C57Bl mice, 7-8 weeks old and weighing 15 to 20 grams were used and treated as it is shown in the following protocol.

Treatment Protocol

Groups: G_1/G_2 treated with EPO alone

G₃ treated with PBS

G₄ treated with Bleomycin alone

G₅ treated with Bleomycin and EPO

Animals:

4-8 per group

Injections:

ip, x2 per week

Agents:

EPO (5-40 u/mouse)

Bleomycin (0.25-0.75 u/mouse)

Sacrificed:

2 wks, 4 wks, 6 wks

Histology

Animals were deeply anesthetized by ketamine/xylazine for sacrifice. The lungs were removed for macroscopic and microscopic analysis. Lung specimens were routinely fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micrometer thick sections were placed on slides and stained with hematoxylin and eosin (H&E), Masson's trichrome stain or factor VIII for qualitative and quantitative morphometric analysis. Cellular alterations of the endothelium were determined by blind analysis of the lung sections using a Nikon microscope.

ICAM-1 Immunostaining and Image Analysis

All samples were routinely processed with 10% formalin and embedded in paraffin. Four-micrometer thick sections from the tissue blocks were placed on PLUS slides. After air drying, tissue sections were deparaffinized and rehydrated before immunostaining. Antigen retrieval was performed by enzyme pretreatment and heating of the tissue sections in a steamer. ICAM-1 specific monoclonal antibody was directed at

the extracellular domain of ICAM-1 antigen and detection of bound antibody was achieved using a commercial kit from Santa Cruz Biotechnology Inc.

Expression of intercellular adhesion molecule-1 (ICAM-1) by endothelial cells was quantified using a Nikon microscope equipped with the SPOT-Advanced image-analysis system and assessing 20 to 30 randomly selected fields via grid analysis. A total of 100 endothelial cells were counted and expressed as positive or negative for immunostaining.

4. Under normal circumstances, the endothelial cells (EC) are spindle-shaped elongated cells. They form the endothelium in the vascular system. The endothelium is a single cell layer separating the lumen of the vessels from the interstitial space. Figure 1 (attached), a lung section stained with H&E, shows a vessel with normal endothelium and endothelial cells. The normal endothelium controls the trafficking of fluids, macromolecules, micromolecules and cells into and out of the lumen. However, when the endothelial cells are activated by various means, including chemotherapeutic agents, they become round and are called prominent endothelial cells. Figure 2 (attached), another lung section stained with H&E, shows a vessel with interrupted endothelium and prominent (activated) endothelial cells. The prominent endothelial cells form a discontinuous endothelium, homeostasis is disturbed and a series of events takes place, which results in tissue damage.

We investigated the structural changes in the endothelium of the lungs caused by bleomycin in animals treated with the drug alone and in combination with EPO at various time intervals as described in the treatment protocol. Lungs obtained from animals treated with various concentrations of EPO or saline had very low levels of prominent endothelial cells, indicating that this treatment does not stimulate the endothelium (Figure 3, attached). Sections of lungs collected from animals injected with bleomycin alone displayed approximately 400% more prominent endothelial cells than those found in lungs collected from mice treated with EPO alone or saline (P<0.05). A 6-fold decrease in prominent endothelial cells was found in lung sections obtained from mice treated with bleomycin and EPO compared to the animals injected with bleomycin alone (p<0.008).

In our studies, two weeks following treatment, the endothelium of bleomycintreated animals expressed high levels of intercellular adhesion molecule-1 (ICAM-1) (Figure 4, attached; bars represent means \pm SE). In 26 assessed fields, 67 ± 11.9 % of the endothelial cells were positive for ICAM-1. However, only 29 ± 6.4 % of lung endothelial cells derived from animals treated with bleomycin and EPO expressed ICAM-1. Thus, EPO induced a 2.7-fold suppression of adhesive molecule expression (P=0.009). A linear relationship between the total cumulative dose of bleomycin and the severity of endothelial cell alterations was seen. While low concentrations of bleomycin had no significant effect on the adhesive molecule over the first two weeks, ICAM-1 expression increased at four weeks following treatment. In 25 fields studied, 53 ± 12.8 % of the pulmonary endothelial cells expressed ICAM-1 (Figure 5, attached; bars represent means \pm SE). In animals injected with EPO and bleomycin, only 20 ± 2.1 % of the endothelial cells expressed ICAM-1 (P=0.007). These results suggest that the ability of EPO to protect endothelium from drug-induced toxicity is mediated by suppressing ICAM-1 expression and inhibiting endothelial cell activation.

5. The endothelium plays an important role in angiogenesis reproduction, embryonic vasculogenesis, wound healing, blood clotting, and in many serious diseases, such as atherosclerosis and cancer. Far from being a passive vessel lining, endothelial cells upon exposure to environmental agents (e.g. chemotherapeutic drugs, radiation, chemicals), biological factors (e.g. cytokines, chemokines, growth factors, LPS) or mechanical means (e.g. cardiac angioplasty) undergo profound structural and functional alterations and in several occasions are unrepairably damaged. This damage can lead to disturbance of vital functions such as hemostasis, inflammatory reactions and immunity.

Our studies indicate that EPO can prevent, protect, and/or repair endothelial damage. The discovery of such means to protect against endothelial injuries or enhance damage recovery will be beneficial for the patient and may decrease the morbidity and/or mortality.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

George	Myouna)	
George Sigouna	as, Ph.D.	

4-15-03 Date